Biogenesis of the Avian Erythroid Membrane Skeleton: Receptor-mediated Assembly and Stabilization of Ankyrin (Goblin) and Spectrin

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ABSTRACT Ankyrin is an extrinsic membrane protein in human erythrocytes that links the $\alpha\beta$-spectrin-based extrinsic membrane skeleton to the membrane by binding simultaneously to the β-spectrin subunit and to the transmembrane anion transporter. To analyse the temporal and spatial regulation of assembly of this membrane skeleton, we investigated the kinetics of synthesis and assembly of ankyrin (goblin) with respect to those of spectrin in chicken embryo erythroid cells. Electrophoretic analysis of Triton X-100 soluble and cytoskeletal fractions show that at steady state both ankyrin and spectrin are detected exclusively in the cytoskeleton. In contrast, continuous labeling of erythroid cells with [^{35}S]methionine, and immunoprecipitation of ankyrin and α- and β-spectrin, reveals that newly synthesized ankyrin and spectrin are partitioned into both the cytoskeletal and Triton X-100 soluble fractions. The soluble pools of ankyrin and β-spectrin reach a plateau of labeling within 1 h, whereas the soluble pool of α-spectrin is substantially larger and reaches a plateau more slowly, reflecting an approximately 3:1 ratio of synthesis of α- to β-spectrin. Ankyrin and β-spectrin enter the cytoskeletal fraction within 10 min of labeling, and the amount assembled into the cytoskeletal fraction exceeds the amount present in their respective soluble pools within 1 h of labeling. Although α-spectrin enters the cytoskeletal fraction with similar kinetics to β-spectrin and ankyrin, and in amounts equimolar to β-spectrin, the amount of cytoskeletal α-spectrin does not exceed the amount of soluble α-spectrin even after 3 h of labeling. Pulse-chase labeling experiments reveal that ankyrin and α- and β-spectrin assembled into the cytoskeleton exhibit no detectable turnover, whereas the Triton X-100 soluble polypeptides are rapidly catabolized, suggesting that stable assembly of the three polypeptides is dependent upon their association with their respective membrane receptor(s). The existence in the detergent-soluble compartment of newly synthesized ankyrin and α- and β-spectrin that are catabolized, rather than assembled, suggests that ankyrin and spectrin are synthesized in excess of available respective membrane binding sites, and that the assembly of these polypeptides, while rapid, is not tightly coupled to their synthesis. We hypothesize that the availability of the high affinity receptor(s) localized on the membrane mediates posttranslationally the extent of assembly of the three cytoskeletal proteins in the correct stoichiometry, their stability, and their spatial localization.

The human erythroid membrane skeleton is a protein complex that plays a dynamic role in maintaining this cell's shape, in re-establishing this shape following deformation, and in influencing the distribution and mobility of membrane components such as integral membrane proteins and phospholipids (reviewed in references 5, 15, 17, 20, 30, 31). Spectrin, a heterodimeric protein, accounts for 75% of the mass of the erythrocyte membrane skeleton (above reviews), and is a component of nonerythroid membrane skeletons as well (10, 16, 18, 19, 25, 36, 38). One membrane-associated receptor for spectrin is another extrinsic membrane protein, ankyrin, which simultaneously binds to spectrin and to the transmembrane anion transporter (6–9, 22, 26, 29, 39; and reviewed in reference 5).

The model of mammalian spectrin being linked to the anion transporter through their mutual binding to ankyrin may also apply to avian erythrocytes, given that their membrane skeletons contain similar polypeptides (21, 38, 40). Like

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mammalian erythrocytes, chicken erythroid cells have a membrane-associated spectrin network containing equimolar proportions of α-spectrin (240,000 M_r) and β-spectrin (220,000 M_r) (21, 38). Chicken erythroid cells also have a hormonally regulated membrane-associated phosphoprotein, goblin (~260,000 M_r), which has similar, but not identical, solubility properties to human erythrocyte ankyrin (1–3, 21, 38). Like human erythrocyte ankyrin, goblin is present in membranes in one-half the quantity of either α- or β-spectrin (21, 38; also, see below). Significantly, globin cross-reacts with antibodies specific for human ankyrin (12; also, see below), forms a complex with spectrin and the anion transporter (37), and has a chymotryptic peptide map homologous to that of mammalian ankyrin (37), further establishing the homology between goblin and human ankyrin. We will, therefore, refer here to goblin as ankyrin because of their apparent structural and functional similarities, though we do not rule out the possibility that human ankyrin and chicken goblin may have unique species-specific properties. Finally, the band 3 and 3.1 proteins in chicken erythrocytes are integral membrane proteins that are glycosylated and largely soluble in Triton X-100 (23, 24, 41), similar to the mammalian anion transport protein (reviewed in reference 15).

In the present study we have investigated the time course of synthesis and assembly of ankyrin. To enable a direct comparison of the coordination of ankyrin assembly relative to spectrin assembly in the same experiment, it was useful to repeat our previous studies on spectrin assembly (12). We find that newly synthesized ankyrin, like β-spectrin, enters a small saturable soluble pool, and that most of it rapidly assembles into the membrane skeleton. Similar to the two spectrin subunits, membrane-associated ankyrin is stable, whereas soluble ankyrin is turned over. On the basis of these data we propose an hypothesis to describe the posttranslational spatial and temporal regulation of assembly of the three membrane skeleton polypeptides.

MATERIALS AND METHODS

Cell Culture and Labeling: Erythroid cells were obtained from 10-d-old chicken embryos, washed in minimal essential medium, and labeled in minimal essential medium supplemented with [^{35}S]methionine (33). In the continuous labeling experiment (see Fig. 2), cells were labeled in the presence of 0.3 μm unlabeled methionine, and in the pulse-chase experiment (see Fig. 3), cells were chased with 240 μm methionine. While the use of low concentrations of methionine in continuous labeling experiments could potentially affect the absolute rate of protein synthesis, under these labeling conditions incorporation is linear for at least 3 h (33). The pulse-chase experiments are also unlikely to be qualitatively influenced by labeling conditions since the cells were labeled for only 10 min in the presence of low methionine concentrations before the chase period. After labeling, cells were fractionated into Triton X-100 soluble and cytoskeletal fractions as previously described (33, 34).

Immunoprecipitation and Quantification: Antibodies directed against electrophoretically purified chicken erythrocyte α- (38) and β-spectrin (36) have been characterized previously. Antibodies against human erythrocyte ankyrin (4), generously supplied by Dr. Vann Bennett, (Johns Hopkins University) were used for immunoprecipitation of goblin. Immunoprecipitation method B (12) was used as previously described (32–34) and immunoprecipitated polypeptides were subjected to SDS 10% or 12.5% PAGE, fluorography, and densitometry (33, 34). The data obtained with a particular antibody are quantitative since the sample sizes and immunoprecipitation conditions are identical for different time points.

RESULTS

Identification of Ankyrin and Spectrin in Fractionated Cells

The human erythroid cytoskeleton can be operationally defined as the residue that is insoluble in the nonionic detergent Triton X-100 (15). Similarly, at steady state, chicken erythroid ankyrin and spectrin are present exclusively in the Triton X-100 insoluble fraction (Fig. 1, lane *2*), and they are not detectable in the soluble fraction (Fig. 1, lane *1*) (see reference 38 for further solubility properties of chicken membrane proteins). Immunoprecipitation of soluble and cytoskeletal fractions with antisera specific for ankyrin (Fig. 1, lanes *3* and *4*), α-spectrin (Fig. 1, lanes *5* and *6*), and β-spectrin (Fig. 1, lanes *7* and *8*) further demonstrates that these proteins are present exclusively in the cytoskeletal fraction at steady state.

After labeling erythroid cells for 1 h with [^{35}S]methionine, followed by lysis in Triton X-100, most of the newly synthesized ankyrin and β-spectrin are found in the cytoskeletal fraction (Fig. 1, lane *11*). In contrast, however, to the steady state distribution of α-spectrin, newly synthesized α-spectrin in molar excess to β-spectrin is present also in the soluble fraction (Fig. 1, lane *10*; see also references 12, 32, and below). In addition, small amounts of ankyrin and β-spectrin are also recovered in the Triton X-100 soluble fraction. A similar distribution of newly synthesized ankyrin and α- and β-spectrin is obtained by examining soluble (Fig. 1, lane *9*) and membrane (Fig. 1, lane *12*) fractions prepared by hypotonic lysis, which indicates that the detergent extraction procedure accurately reflects the membrane association of ankyrin and equimolar proportions of α- and β-spectrin, and the soluble nature of the excess α-spectrin, β-spectrin, and ankyrin (see also reference 12 for α- and β-spectrin). Newly synthesized ankyrin (Fig. 1, lane *13*), α-spectrin (Fig. 1, lane *14*), and β-spectrin (Fig. 1, lane *15*) can be identified more conclusively in both the soluble (not shown) and cytoskeletal (Fig. 1) fraction by immunoprecipitation as shown in Fig. 1; hence this approach was used to obtain the data shown in Figs. 2 and 3.

Quantification by densitometry of Coomassie-Blue-stained gels reveals that at steady state the relative abundance of ankyrin to α-spectrin to β-spectrin is approximately 0.5:1.3:1.0 in both detergent-extracted cytoskeletons and membranes prepared by hypotonic lysis, assuming equal efficiency of dye binding to all three polypeptides. Quantification of fluorographs of cytoskeletons prepared from cells labeled with [^{35}S]methionine for 1 h reveals an apparent stoichiometry of newly synthesized ankyrin to α-spectrin to β-spectrin of 0.7:0.8:1.0 without taking into consideration any possible differences in methionine content.

Most Newly Synthesized Ankyrin Is Rapidly Assembled

We have previously established that α-spectrin is synthesized in molar excess of β-spectrin in vivo and in vitro but that α-spectrin in molar excess to β-spectrin does not assemble into the membrane skeleton (12, 32). To determine whether the partitioning of newly synthesized ankyrin into the soluble and cytoskeletal fractions was similar to either α- or β-spectrin, we labeled chicken embryo erythroid cells with [^{35}S]-methionine for various times followed by detergent extraction and immunoprecipitation. Quantification of the immunoprecipitates reveals that small amounts of ankyrin and β-spectrin, and greater amounts of α-spectrin, are present in the Triton X-100 soluble fraction (Fig. 2*A*). The amount of labeled ankyrin and β-spectrin in the soluble fraction reaches a pla-

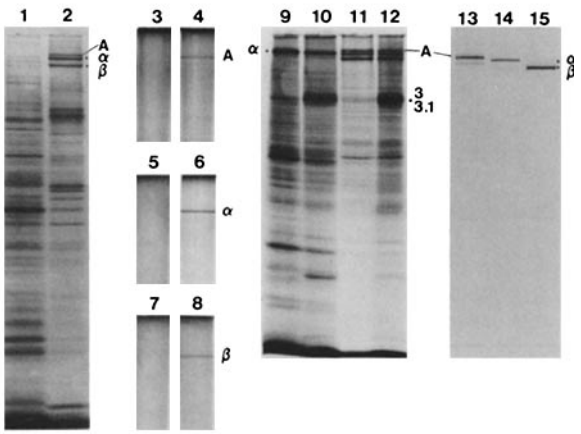

FIGURE 1 Identification of ankyrin and spectrin in fractionated cells. Erythroid cells from 10–11-d-old chicken embryos were fractionated into Triton X-100 soluble (lane *1*) and cytoskeletal fractions (lane *2*) (33) and aliquots were separated on SDS/12.5% polyacrylamide gels before staining with Coomassie Blue to identify the cytoskeletal proteins (*A*, ankyrin; *α*, *α*-spectrin; *β*, *β*-spectrin). Aliquots of fractionated cells were also used for immunoprecipitation of ankyrin in the soluble (lane *3*) and cytoskeletal (lane *4*) fractions, of *α*-spectrin in the soluble (lane *5*), and cytoskeletal fractions (lane *6*), and of *β*-spectrin in the soluble (lane *7*) and cytoskeletal (lane *8*) fractions to illustrate the steady state distribution of these polypeptides. The immunoprecipitates were subjected to SDS PAGE and stained with Coomassie Blue—only the upper portion of the gel is presented (see also lanes *13–15*). To determine the extent of assembly of newly synthesized membrane-skeleton polypeptides, we labeled 10–11-d-old erythroid cells for 1 h (35°C) with [^{35}S]-methionine (33) before separation by one of two methods into soluble and membrane-skeleton fractions. One aliquot of cells was lysed in a buffer containing Triton X-100 and separated by centrifugation into a detergent soluble fraction (lane *10*) and a cytoskeletal fraction (lane *11*). Another aliquot of labeled cells was lysed in five vol of a hypotonic buffer (4 mM $NaPO_4$, 4 mM EGTA, 1 mM EDTA, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and separated by centrifugation (12,500 *g* for 5 min) into a soluble (lane 9) fraction and a membrane/nuclei fraction (lane *12*). Whereas both the cytoskeletal (lane *11*) and membrane (lane *12*) fractions contain most of the newly synthesized ankyrin (a) and *β*-spectrin (*β*), *α*-spectrin (*α*) is partitioned between both soluble (lanes *9* and *10*) and insoluble (lanes *11* and *12*) fractions. The anion transporter (bands 3, 3.1) is an integral membrane protein and is, therefore, present in membrane fractions (lane *12*), though the majority of these polypeptides are not associated with the membrane skeleton (reviewed in references 5, 15) and hence are extracted by Triton X-100 (lane *10*). Lanes *13–15* are immunoprecipitates of [^{35}S]methionine-labeled, Triton X-100 cytoskeletal fractions (lane *11*) with antibodies to ankyrin (lane *13*), *α*-spectrin (lane *14*) and *β*-spectrin (lane *15*).

teau within 1 h of labeling, suggesting that these polypeptides have small, saturable soluble pools (Fig. 2*A*). The proportion of total labeled ankyrin in the soluble fraction is 46% at 60 min, and 15% at 120 min, whereas the proportion of total labeled *β*-spectrin in the soluble fraction is 37% at 60 min and 14% at 120 min. In contrast, the labeled *α*-spectrin in the soluble fraction does not reach a plateau before 2 h of labeling, consistent with our previous demonstration of a large soluble pool of *α*-spectrin (12). On the other hand, ankyrin, and both spectrin subunits, enter the cytoskeleton within 30 min of labeling in this experiment (within 10 min when more [^{35}S]-methionine is used, e.g., Fig. 3) and accumulate in an approximately linear fashion for the duration of the experiment (Fig. 2*B*). The relative distribution between the soluble and cytoskeletal fractions of newly synthesized ankyrin thus qualitatively resembles that of *β*-spectrin, and is qualitatively dissimilar from the distribution of *α*-spectrin. Since *α*-spectrin

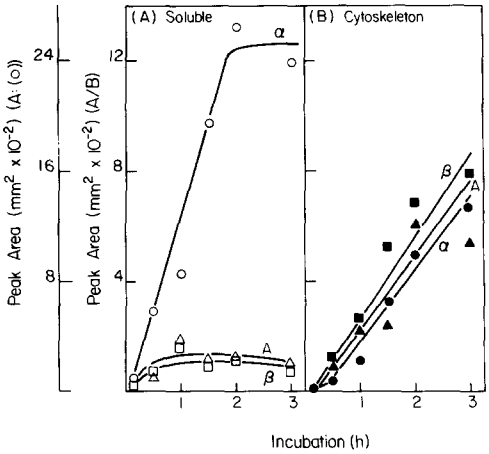

FIGURE 2 Most newly synthesized ankyrin is rapidly assembled. Erythroid cells (1% vol/vol in minimal essential media containing 5% fetal calf serum) from 10-d-old chicken embryos were labeled continuously for 3 h at 35°C with [^{35}S]methionine (220 *μ*Ci/ml, 1,200 Ci/mmol) and 0.3 *μ*M unlabeled methionine (33). At the indicated intervals, aliquots were fractionated into Triton X-100 soluble (*A*) and cytoskeletal (*B*) fractions (33). Equivalent volumes of these fractions were used for immunoprecipitation of ankyrin (△, ▲,) *β*-spectrin (□, ■), and *α*-spectrin (○, ●), followed by SDS PAGE, fluorography, and densitometry. Fluorographs were exposed for 18 h, scanned with a recording densitometer, and peak areas were integrated by computer. The outer scale on the ordinate refers only to the soluble *α*-spectrin (○) in *A*. The inner scale refers to all other curves in *A* and *B*.

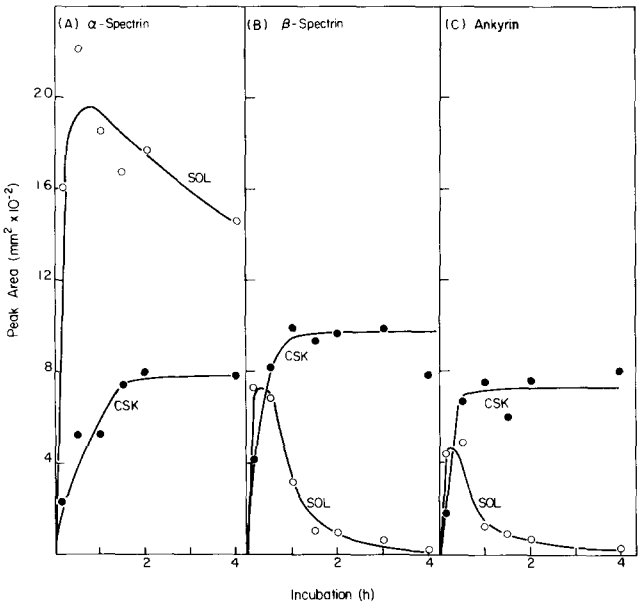

FIGURE 3 Lability of the soluble form of newly synthesized ankyrin. In the same experiment as Fig. 2, erythroid cells were labeled with [^{35}S]methionine for 10 min, then unlabeled methionine was added to 0.24 mM and the cells incubated for 4 h (35°C). Aliquots of cells were periodically separated into Triton X-100 soluble (○) and insoluble (●) fractions, and processed for immunoprecipitation of *α*-spectrin (*A*), *β*-spectrin (*B*), and ankyrin (C), gel electrophoresis, and fluorography. Data represent the peak areas of fluorographs exposed for 18 h.

has a large soluble pool, this indicates that α-spectrin is synthesized in several-fold excess relative to the availability of its respective cytoskeletal binding sites. Ankyrin and β-spectrin on the other hand, since they have relatively smaller saturable soluble pools, appear to be synthesized in smaller excess of their respective cytoskeletal binding sites.

Lability of the Soluble Form of Newly Synthesized Ankyrin

To investigate the stability of ankyrin relative to the spectrin subunits in the soluble and cytoskeletal fractions, we labeled erythroid cells with [^{35}S]methionine for 10 min, then prevented further labeling by chasing with a vast excess of unlabeled methionine for 4 h. Cells were periodically fractionated and ankyrin and spectrin were quantified by immunoprecipitation from the same soluble and cytoskeletal fractions. We first demonstrated that labeled spectrin behaves as previously described (12). Briefly, approximately equimolar proportions of labeled α-spectrin (Fig. 3*A*) and β-spectrin (Fig. 3*B*) enter the cytoskeletal fraction and reach a plateau by 1 h following commencement of the chase. There is little detectable turnover of this spectrin in the cytoskeletal fraction during the 4 h chase period (Fig. 3) and even up to 8 h (data not shown). With regard to soluble spectrin, there is more soluble α-spectrin than soluble β-spectrin. The β-spectrin does not accumulate in the soluble fraction since it turns over rapidly during the chase period, with a half life of ~45 min (Fig. 3*B*). Soluble labeled α-spectrin also turns over, with a half life of ~4 h (Fig. 3*A*).

Strikingly, labeled ankyrin behaves in a qualitatively similar fashion to β-spectrin during the chase period. Most of the newly synthesized ankyrin enters the cytoskeleton within 1 h of initiation of the chase, and this cytoskeletal ankyrin exhibits little detectable turnover (Fig. 3*C*). On the other hand, the small amount of soluble ankyrin turns over rapidly with a half-life of ~45 min (Fig. 3*C*). At least some of the soluble labeled spectrin and ankyrin appear to be catabolized rather than slowly assembled into the cytoskeletal fraction during the chase period because decreases in the amount of soluble polypeptides do not yield proportional increases in cytoskeletal polypeptides, as would be expected if a precursor-product relationship existed without catabolism. These data indicate, therefore, that assembly onto the cytoskeleton confers stability to ankyrin and α- and β-spectrin, whereas a mechanism exists for the selective catabolism of unassembled, soluble cytoskeletal polypeptides. These data also indicate that there is not a strict coupling between synthesis and assembly of the two spectrin subunits and ankyrin, since not all newly synthesized polypeptides are assembled.

DISCUSSION

Posttranslational Assembly of Ankyrin and Spectrin

In mature chicken and mammalian erythrocytes the spectrin-based membrane cytoskeleton is segregated in the cytoplasm and localized exclusively under the plasma membrane. The present report extends our earlier work on the assembly of this cytoskeleton (12, 32) in that we have now followed the kinetics of synthesis and assembly of ankyrin relative to those of spectrin. The evidence presented here and previously (12, 40) demonstrate that the proteins that comprise the membrane skeleton, including ankyrin, the two spectrin subunits, and the anion transporter, are synthesized simultaneously in developing erythroid cells. Since at least spectrin is capable of interacting in solution to form higher order structures (35), the observed simultaneous synthesis of membrane skeleton proteins could result in the assembly of polypeptides at inappropriate sites. During erythroid development, however, assembly appears to be regulated in such a way that stable higher order structures form only at the plasma membrane. This regulation of assembly does not appear to involve coordinate regulation of transcription or translation of precise amounts of the anion transporter with respect to ankyrin and the two spectrin subunits, since the reduction in the expression of these proteins upon terminal differentiation is asynchronous (40). The regulation of assembly of the membrane skeleton is probably not achieved cotranslationally either, since the polypeptides that comprise the cytoplasmic domain of the membrane skeleton are synthesized on membrane-free polyribosomes (27, 28), whereas the anion transporter, which serves as an ultimate membrane receptor for the membrane skeleton, is cotranslationally inserted in the endoplasmic reticulum and vectorially transported to the plasma membrane (13, 14). That membrane skeleton components do not assemble during translation was directly shown by experiments using a cell-free system to demonstrate that both α- and β-spectrin (32) and ankyrin (unpublished observations) will bind posttranslationally to inverted erythrocyte vesicles. Furthermore, α-spectrin nascent polypeptides are released from Triton X-100 cytoskeletons by puromycin, suggesting that they do not assemble on the membrane cotranslationally (11).

One of the most important observations reported here for ankyrin and previously for α- and β-spectrin (12) is that all three polypeptides are synthesized in excess of the amount of each that is assembled. While the amount of ankyrin and β-spectrin synthesized is in small excess of the amount assembled, the amount of α-spectrin that is synthesized exceeds by several fold the amount that is assembled. Since ankyrin and spectrin are synthesized in excess of the amounts that are assembled in vivo (present study and reference 12) or in vitro (32), and since spectrin nascent chains on polyribosomes are not bound to the cytoskeleton, collectively these observations argue strongly that the assembly of the proper stoichiometry of ankyrin and spectrin occurs posttranslationally. Our observation that only small amounts of newly synthesized ankyrin and β-spectrin are present in the Triton X-100 soluble fraction may underestimate the actual proportion of unassembled ankyrin and β-spectrin if these polypeptides form a higher order structure that is insoluble under our extraction conditions yet not associated with the membrane skeleton. Therefore, these molecules may be synthesized in even greater excess relative to the availability of cytoskeletal binding sites than is indicated in Fig. 2. Finally with regard to the insolubility in Triton X-100 of newly synthesized ankyrin and spectrin, it is possible that the insoluble nature of a large proportion of these polypeptides may reflect their association with the anion transporter soon after the synthesis of the anion transporter on the rough endoplasmic reticulum.

Even though assembly occurs posttranslationally, as shown here, the assembly process is efficient and rapid since both spectrin subunits and ankyrin are detected in the cytoskeletal fraction within minutes after synthesis; additionally the ratio of newly assembled polypeptides closely resembles that at steady state. Since there are always some unassembled polypeptides that are turned over in the soluble compartment and

that therefore do not appear to serve as precursors for assembly, the assembly of ankyrin and the spectrins, even though an efficient and rapid process, is not coupled to their synthesis.

Stability of Assembled and Catabolism of Unassembled Ankyrin and Spectrin

One more important observation to emerge from the studies reported here for ankyrin and previously for α- and β-spectrin (12) is that the newly assembled polypeptides onto the membrane-skeleton are resistant to catabolism, whereas a mechanism exists for the selective degradation of soluble ankyrin and spectrin. An implication of these observations is that none of the three polypeptides can stabilize each other in the soluble compartment, and even if they could associate into a higher order complex, they would still be degraded. Perhaps this may be a preventive mechanism that the cell has to ensure the accumulation of the assembled complex only at the correct cytoplasmic site. However, the mechanism by which the membrane stabilizes the three polypeptides is presently unknown. From in vitro reconstitution experiments the association between ankyrin and the anion transporter has been shown to be of high affinity ($K_d = 5 \times 10^{-9}$ M) (9, 22); as is the interaction of spectrin with ankyrin ($K_d = 5 \times 10^{-8}$ M) (8, 39). Thus we may hypothesize that high affinity interactions lead to the sequential binding of ankyrin to the anion transporter and $\alpha\beta$-spectrin onto ankyrin, resulting in a stabilization of the assembled complex against catabolism. In this manner only the ultimate receptor at the membrane can confer stability to the whole complex.

Limiting Steps in the Assembly of Ankyrin and Spectrin

The synthesis of ankyrin and α- and β-spectrin in excess of the amounts of each polypeptide that are insoluble in Triton X-100 argues that all three polypeptides are synthesized in excess of the availability of their respective binding sites on the membrane. This suggests that the availability of components in the assembly complex proximal to the membrane (e.g., the anion transporter) sequentially limit the extent of assembly of those components distal to the membrane (e.g., ankyrin, α- and β-spectrin). Starting at the distal (cytoplasmic) end of the complex, α-spectrin clearly does not limit the extent of assembly of β-spectrin or ankyrin since it does not provide any known binding site through which these polypeptides bind to the membrane and since it is synthesized in a severalfold excess over both β-spectrin and ankyrin. On the other hand in vitro reconstitution studies using a cell-free system have shown that β-spectrin limits the extent of assembly of α-spectrin, since only α-spectrin in proportions equimolar to β-spectrin are assembled (32). Ankyrin, in turn, may limit the extent of assembly of β-spectrin onto the membrane since not all newly synthesized β-spectrin is assembled.

Analogous arguments also apply to the anion transporter limiting the extent of ankyrin assembly, and hence the overall extent of assembly of all three cytoplasmic polypeptides. In both mammalian (reviewed in reference 15) and avian (see above and reference 38) erythroid cell membranes, the anion transporter is present in a several-fold excess to the amount of ankyrin (goblin) and hence it is potentially nonlimiting in the extent of assembly. Three lines of evidence, however, indicate that the anion transporter limits the overall extent of assembly. The first is that in reconstitution experiments using spectrin depleted inside-out vesicles, only a small subset of anion transporters may be competent to bind ankyrin (reviewed in references 5, 15). One of the reasons for this may be that the anion transporter exists in the form of oligomers in the membrane that may reduce the sites available for ankyrin binding. The second line of evidence for the anion transporter limiting assembly is that even though the anion transporter exists in embryonic erythroid cell membranes in several-fold excess over ankyrin, and is synthesized in several-fold excess over ankyrin, not all of newly synthesized ankyrin assembles onto the membrane in vivo. The third line of evidence is that membranes from embryonic erythroid cells active in the synthesis of all membrane-skeleton polypeptides in vivo will bind only very small quantities of newly synthesized spectrin (32) or ankyrin (unpublished observations) in a cell-free reconstituted system. All these lines of evidence argue that not only at steady state in the membrane, but also upon insertion of newly synthesized polypeptides into the membrane, only a subset of the anion transporters are competent to bind to ankyrin and hence limits the overall extent of assembly of the complex.

Receptor-mediated Assembly and Stabilization of Ankyrin and Spectrin

The above data on the synthesis, assembly, and turnover of ankyrin and spectrin enable us to propose the following working hypothesis for the posttranslational assembly of the erythroid cell membrane-skeleton (Fig. 4). The assembly of the constituent polypeptides of the membrane skeleton requires both spatial and temporal regulation. The spatial localization of spectrin and ankyrin is regulated predominantly by the vectorial flow to the plasma membrane of newly synthesized anion transporters, which provide localized high affinity binding sites for ankyrin and, in turn, spectrin. The temporal regulation of assembly is achieved by the simultaneous synthesis of all components to be assembled; the correct stoichiometry of each polypeptide in the forming structure is determined posttranslationally by the availability of high affinity binding sites. The ultimate receptor in the membrane, presumed to be the anion transporter, limits the overall extent of assembly of ankyrin and spectrin. Ligands (spectrin and ankyrin) that are assembled are resistant to catabolism, whereas unassembled spectrin and ankyrin are catabolized, thus preventing the accumulation and assembly of reactants at inappropriate sites.

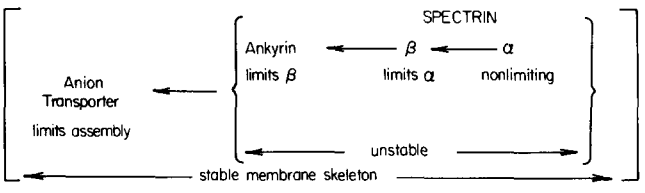

FIGURE 4 The receptor-mediated assembly and stabilization hypothesis. The anion transporter mediates the spatial organization of the membrane skeleton since it is localized at the plasma membrane and serves as a high affinity binding site for ankyrin. Ankyrin, in turn, provides a localized high affinity binding site for equimolar proportions of α- and β-spectrin. Since not all newly synthesized membrane skeleton polypeptides are assembled (e.g., two-thirds of newly synthesized α-spectrin are not assembled), the availability and affinities of receptors and ligands, rather than transcriptional controls, determines the stoichiometry of the constituent polypeptides of the membrane skeleton. Since only assembled polypeptides are resistant to catabolism, the association of a ligand with its receptor mediates stability. Further details are given in the text.

The physiological significance of this hypothesis is that it explains how the cell assembles the membrane skeleton in the shortest period of time (all components are synthesized concurrently), while simultaneously ensuring that assembled components accumulate only at the plasma membrane (high affinity binding sites are present at the membrane, and binding confers stability to ankyrin and spectrin). The key feature of this hypothesis is that it explains how at steady state the relative proportions of membrane skeleton proteins are determined posttranslationally by the amount of each protein binding to their respective membrane receptor, and not by the amount of each protein synthesized.

It should be realized that even though this hypothesis is consistent with all experimental evidence we have obtained thus far, many details of the assembly process are unknown. As mentioned earlier, we do not know whether ankyrin and spectrin assemble onto newly synthesized anion transporters and are cotransported on vesicles to the plasma membrane, or whether all components assemble at the plasma membrane. Second, spectrin may associate with the membrane skeleton by interacting with nonankyrin binding sites such as preexisting, already assembled spectrins, or band 4.1 proteins. The presence of some unassembled spectrin may, therefore, reflect the inefficiency of forming spectrin oligomers by high spectrin concentrations and with low affinities for each other (35), or serve a physiologically significant role by increasing the rate of spectrin assembly. The potential importance of high and low affinity interactions has been previously used to explain the assembly of the spectrin based membrane-skeleton (35). From what we have discussed above, however, it is evident that an hypothesis on the assembly of the membrane-skeleton has to account not only for the site of assembly of this network, but also for the observation that the cytoplasmic components of this network are synthesized in excess of their membrane-binding sites, and for the observation that only those ankyrin and α- and β-spectrin polypeptides that assemble onto the membrane are stabilized against catabolism.

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